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ORIGINAL ARTICLE

Elevated serum biotinidase activity in hepatic glycogen storage disorders–A convenient biomarker

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Summary An elevated serum biotinidase activity in patients with glycogen storage disease (GSD) type Ia has been reported previously. The aim of this work was to investigate the specificity of the phenomenon and thus we expanded the study to other types of hepatic GSDs. Serum biotinidase activity was measured in a total of 68 GSD patients and was compared with that of healthy controls (8.7 ± 1.0; range 7.0–10.6 mU/ml; $n = 26$). We found an increased biotinidase activity in patients with GSD Ia (17.7 ± 3.9; range: 11.4–24.8; $n = 21$), GSD I non-a (20.9 ± 5.6; range 14.6–26.0; $n = 4$), GSD III (12.5 ± 3.6; range 7.8–19.1; $n = 13$), GSD VI (15.4 ± 2.0; range 14.1–17.7; $n = 3$) and GSD IX (14.0 ± 3.8; range: 7.5–21.6; $n = 22$). The sensitivity of this test was 100% for patients with GSD Ia, GSD I non-a and GSD VI, 62% for GSD III, and 77% for GSD IX, indicating reduced sensitivity for GSD III and GSD IX, respectively. In addition, we found elevated biotinidase activity in all sera from 5 patients with Fanconi–Bickel Syndrome (15.3 ± 3.7; range 11.0–19.4). Taken together, we propose serum biotinidase as a diagnostic biomarker for hepatic glycogen storage disorders.

Abbreviations

BTD	biotinidase
FBS	Fanconi–Bickel syndrome
GSD	glycogen storage disease
LSD	lysosmal storage disorder

Introduction

Several studies have shown that serum biotinidase is markedly elevated in patients with GSD Ia (OMIM 232200; Burlina et al 1996; Hug et al 1994; Saltik et al 2000; Wolf et al 2003). Biotinidase catalyses the hydrolysis of biocytin to biotin and is responsible for recycling of endogenous biotin (Hymes and Wolf 1996). The enzyme is synthesized mainly by the liver and then secreted into the blood (Grier et al 1990). Biotinidase is not directly related to glycogen metabolism and, to date, the cause of increased activity in GSD type Ia patients has remained unclear. While previous reports suggested that sensitivity in GSD Ia patients is high, there has been limited information on the specificity of these findings. In this study, we evaluated serum biotinidase activity in a significant number of patients with enzymatically and/or molecular genetically proven GSDs including GSD I non-a (OMIM 232220), GSD III (OMIM 232400), GSD VI (OMIM 232700) and GSD IX (OMIM 306000), as well

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References to electronic databases: Glycogen storage disease (GSD) Ia: OMIM 232200. GSD I non-a: OMIM 232220. GSD III: OMIM 232400. GSD VI: OMIM 232700. GSD IX: OMIM 306000. Fanconi–Bickel syndrome: OMIM 227810. Cystic fibrosis: OMIM 602421. Gaucher disease: OMIM 230800. Niemann–Pick disease (NP) type A: OMIM 257200. NP type C: OMIM 257220.

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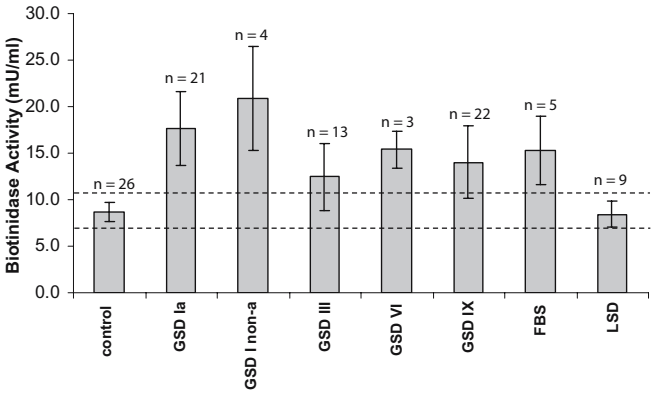

Fig. 1 Summary of biotinidase activities from controls, patients with hepatic GSD and LSD patients. Means and SD are indicated. Lysosomal storage disorders include Niemann–Pick A ($n=2$), Niemann–Pick C ($n=2$), and Gaucher disease ($n=5$). The dashed lines represent the range in controls (7.0–10.6 mU/ml). FBS, Fanconi–Bickel syndrome; GSD, glycogen storage disorders; LSD, lysosomal storage disorders

as patients with Fanconi–Bickel syndrome (FBS), which is characterized by the failure to transfer glucose across plasma membranes due to congenital deficiency of the glucose transporter-2 (OMIM 227810).

Patients and methods

Patients

All patients had a biochemically proven GSD. In most cases elevated glycogen concentration in the liver was demonstrated. They were assigned to GSD types I a, I non-a, III, VI and IX and to FBS according to typical results of enzymatic and/or molecular genetic studies (see Tables 2 and 3).

Serum biotinidase activity

Whole blood samples were collected and centrifuged and the resulting sera were frozen at −20°C until analysis. Serum biotinidase activity was determined by a colorimetric assay using biotinyl-*p*-aminobenzoate as substrate (Wolf et al 1983). The mean biotinidase activity in serum from healthy individuals was determined to be 8.7 ± 1.0 mU/ml (range 7.0–10.6; $n=26$) and was age-independent. The inter-assay coefficient of variation for the activity in a control serum was 7.4% ($n=10$).

Quantitative determination of liver glycogen

Liver glycogen was analysed using anthron reagent (Seifter et al 1950).

Determination of liver enzyme activities

Glucose-6-phosphatase was measured by colorimetric assessment of inorganic phosphate released from glucose 6-phosphate (Hers 1964). To determine amylo-1, 6-glucosidase activity, ^{14}C-labelled glucose was incorporated into glycogen by the *in vitro* reverse activity, followed by precipitation and measurement of radiolabelled glycogen (Van Hoof 1967). Total phosphorylase activity was determined in the presence of adenosine monophosphate by colorimetric measurement of inorganic phosphate released from glucose-l-phosphate (Hers 1964). Phosphorylase b-kinase activity was measured as described using exogenous phosphorylase b from rabbit muscle (Lederer et al 1975).

Mutation analysis

Molecular genetic analysis of affected genes (*G6PC*, *G6PT1*, *AGL*, *PYGL*, *PHKA2*, *GLUT2 [SLC2A2]*) was performed by sequencing PCR products containing exons and adjacent intronic segments of these genes and comparison with the known reference sequences. In general, these PCR products were generated by specific sense and antisense primers from genomic DNA extracted from lymphocytes. Initially, sequencing was performed using the Thermo-Sequenase cycle sequencing kit (Amersham/Buchler, Wenden, Germany) with

Table 1 Serum biotinidase activity in controls and patients with hepatic storage disorders

Disorder	*n*	Biotinidase activity (mU/ml)		Sensitivity (%)
		Mean ± SD	Range	
Control	26	8.7 ± 1.0	7.0–10.6	
GSD Ia	21	17.7 ± 3.9	11.4–24.8	100
GSD I non-a	4	20.9 ± 5.6	14.6–26.0	100
GSD III	13	12.5 ± 3.6	7.8–19.1	62
GSD VI	3	15.4 ± 2.0	14.1–17.7	100
GSD IX	22	14.0 ± 3.8	7.5–21.6	77
Fanconi–Bickel syndrome	5	15.3 ± 3.7	11.0–19.4	100

Table 2 Biotinidase activity in hepatic glycogen storage diseases (GSD) and Fanconi–Bickel syndrome (FBS)

Disorder	Patient	Biotinidase activity (mU/ml) Control mean = 8.7 (range 7.0–10.6; $n = 26$)	Glycogen (g/100 g liver) Control mean = 4.6 (range 2.4–6.4; $n = 12$)	Enzyme	Gene locus
GSD Ia				**Glc-6-Pase (U/g), liver** Control: mean = 6.3 (range: 3.4–9.6; $n = 17$)	***G6PC***
	1	14.3	10.8	0	c.326C > T/c.272G > C
	2	15.8, 12.5 (1 y)[a]	8.8	0.1	c.326C > T/c.1118C > T
	3	19.9	8.7	0	c.326C > T/c.1118C > T
	4	20.3	9.5	0.5	c.407G > A/c.675A > G
	5	16.0	5.6	0	c.641G > C/c.588G > A
	6	24.8, 15.1 (2 y)[a]	11.6	0.3	c.579G > A (homoz.)
	7	18.2	6.6	0.1	c.192A > T/c.326C > T
	8	23.2	7.9	0.0	c.326C > T/c.158delC
	9	21.8	16.5	0.2	c.326C > T/c.272G > C
	10	20.1, 15.1 (2 y)[a]	10.8	0.0	c.588G > A/c.962C > T
	11	18.0	11.3	0.4	c.326C > T/n.i.
	12	15.2	n.d.	n.d.	c.326C > T/c.1118C > T
	13	21.6	n.d.	n.d.	c.326C > T/c.G641 > C
	14	14.6	5.0	0.02	c.326C > T/n.i.
	15	11.8	11.7	0.5	
	16	11.4	7.9	0.5	
	17	13.5	16.4	0.02	
	18	19.9	8.4	0.4	
	19	22.3	9.8	0.0	
	20	14.6	10.2	0.3	
	21	13.6	7.2	0.3	
GSD I non-a					***G6PT1***
	1	25.2, 24.3 (3 y)[a]	8.4		c.1211delCT (homoz.)
	2	17.6, 21.8 (4 y)[a]	15.2		c.250T > A (homoz.)
	3	26.0	6.3		c.1211delCT/c.250T > A
	4	14.6	n.d.		c.1211delCT/c.1348G > A
GSD III				**Amylo-1,6-glucosidase, liver (U/g)** Control mean = 214 (range 157–280; $n = 16$)	***AGL***
	1	15.4	14.0	1.6	c.987C > T/long range del.
	2	14.7, 16.1 (2 y)[a]	17.6	13.0	[c.4459C > T/IVS6 + 3a > g] (homoz.)
	3	15.2	9.7	0.2	c.3980G > A (homoz.)
	4	15.4, 15.7 (8 y)[a]	16.9	1.2	c.1992-3insCG (homoz.)
	5	13.8	8.5	0.0	c.3980G > A (homoz.)
	6	11.9	13.8	0.0	
				Amylo-1,6-glucosidase, Ec (U/g Hb) Control mean = 2.3 (range 0.9–4.2; $n = 17$)	
	7	19.1	n.d.	0.1	c.2497C > T (homoz.)
	8	13.6	n.d.	0.6	c.1589C > G/c.2590C > T
GSD VI				**Phosphorylase (U/g), liver** Control mean = 22.3 (range 11.3–31.3; $n = 16$)	
	1	14.1	26.8	5.3	
	2	17.7	7.4	6.1	
	3	14.4	9.1	3.9	

Table 2 Continued

Disorder	Patient	Biotinidase activity (mU/ml) Control mean = 8.7 (range 7.0–10.6; $n = 26$)	Glycogen (g/100 g liver) Control mean = 4.6 (range 2.4–6.4; $n = 12$)	Enzyme	Gene locus
GSD IX				**Phosphorylase b-kinase, liver (U/g)** Control mean = 83.5 (range 55.5–112.8; $n = 7$)	***PHKA2***
	1	17.2, 16.3 (7 y)[a]	13.9	5.5	
	2	21.6	14.3	22.8	
	3	14.5	8.8	28.9	
	4	16.4	8.7	16.4	
	5	13.3, 16.6 (1 y)[a], 14.5 (7 y)[a]	11.3	0.0	
				Phosphorylase b-kinase, Ec (U/g Hb) Control mean = 10.2 (range 6.3–12.4; $n = 9$)	
	6	16.9	8.9	19.2	c.557G > A (hemiz.)
	7	16.4	n.d.	0.1	c.2747G > T (hemiz.)
	8	10.9	12.4	2.8	
	9	16.3	n.d.	0.7	
	10	16.8	n.d.	0.6	
	11	18.3	n.d.	0.4	
	12	12.7	n.d.	0.7	
				Phosphorylase b-kinase, liver/*Ec	
	13	14.7	7.4	6.1/*2.7	c.740C > G (hemizyg.)
	14	14.7	17.3	24.9/*1.4	
	15	19.8	11.0	6.8/*0.4	
	16	18.0	11.0	6.9/*1.3	
	17	16.1	9.9	40.0/*8.0	
Fanconi–Bickel Syndrome					***GLUT2***
	1	11.0	n.d.		M350 fs370X/R365X
	2	11.9	n.d.		L368 fs391X (homoz.)
	3	16.8	n.d.		c.884A > G (homoz.)
	4	19.4	n.d.		V45 fs74X (homoz.)
	5	17.4	n.d.		V45 fs74X (homoz.)

n.d. = not determined; n.i. = not identified.
Ec, erythrocytes; Glc-6-Pase, glucose-6-phosphatase; Hb, hemoglobin.
[a] Biotinidase activity obtained after the first measurement (years).

^{33}P-labelled terminators, which was later changed to an automated non-radiographic method using the BigDye 3.1 chemistry and an ABI Prism 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Likewise, the biotinidase gene (*BTD*) was analysed in three patients with GSD III and IX, respectively, and in five individuals with enzymatically proven biotinidase deficiency.

Results and discussion

Elevated serum biotinidase activity

Based on published data on elevated serum biotinidase activity in GSD Ia patients, we speculated that biotinidase might also be elevated in additional GSD types. As in other inborn errors of metabolism, this

phenotype is probably not a reflection of the disease *per se*, but rather a consequence of a specific metabolic derangement. To test this, we measured biotinidase in sera from GSD patients with other defects of enzyme or transport proteins involved in the glycogenolytic pathway. To validate our data, GSD type Ia patients were also included. The results are summarized in Fig. 1 and Tables 1 and 2. All subgroups of hepatic glycogen storage diseases showed a marked increase of serum biotinidase activity. On average, the increase ranged from 1.5-fold (GSD type III) up to 2.4-fold (GSD type I non-a). Interestingly, we also found about 1.8-fold elevated serum biotinidase activity in five patients with Fanconi–Bickel syndrome.

Sensitivity

Using mean +2SD as the cut-off value, sensitivity of the biotinidase test was 100% for patients with GSD Ia, GSD I non-a and GSD VI. For GSD I non-a ($n = 4$) and GSD VI ($n = 3$) patients, however, more sera will need to be analysed to confirm these findings.

The sensitivity for GSD type III and IX was 62% and 77%, respectively. This implies that we identified patients with biotinidase activities within the normal range (Table 2). Defects at these enzymatic steps might cause milder metabolic consequences that are not sufficient to induce the mechanisms eventually resulting in elevated biotinidase activity. In this

Table 3 GSD III and GSD IX patients with normal biotinidase activity

Disorder	Patient	Biotinidase activity (mU/ml) Control mean = 8.7 (range: 7.0–10.6; $n = 26$)	Glycogen (g/100 g liver) Control mean = 4.6 (range: 2.4–6.4; $n = 12$)	Enzyme	Gene locus	*BTD* gene
GSD III				**Amylo-1,6-glucosidase, liver (U/g)** Control mean = 214 (range 157–280; $n = 16$)	***AGL***	
	1	7.8, 9.3 (1 y)[a]	5.7	0.8	c.2929C > T/c.4221delA	WT/WT
	2	9.5	14.0	49.0	n.d.	n.d.
				Amylo-1,6-glucosidase, Ec (U/g Hb) Control mean = 2.3 (range 0.9–4.2; $n = 17$)		
	3	8.0		0.0	c.1020delA (homoz.)	WT/WT
	4	8.2		0.03	c.IVS21 + 1G > A/ c.3216-7delGA	n.d.
	5	9.6		0.2	n.d.	n.d.
GSD IX				**Phosphorylase b-kinase, liver (U/g)** Control mean = 83.5 (range 55.5–112.8; $n = 7$)	***PHKA2***	
	1	10.3	13.4	30.5	c.557G > A (hemiz.)	n.d.
				Phosphorylase b-kinase, Ec (U/g Hb) Control mean = 10.2 (range 6.3–12.4; $n = 9$)		
	2	9.7	n.d.	0.0	c.265dupC/ c454C > T	WT/WT
	3	8.1, 10.7 (1 y)[a]	n.d.	0.5	c.1643-62del20 (hemiz.)	n.d.
	4	7.5	n.d.	0.6	n.d.	n.d.
	5	9.4	n.d.	0.5	n.d.	n.d.

n.d. = not determined.
Ec, erythrocytes; Hb, hemoglobin; WT, wild-type.
[a] Biotinidase acitivity obtained after the first measurement (years).

context, however, it is interesting that we found no correlation between the residual activities of the glycogenolytic enzymes and biotinidase activity *in vitro* (see Tables 2 and 3). Taken together, these findings indicate that biotinidase is a less sensitive biomarker for GSD III and GSD IX.

In order to exclude genetic causes of a restricted increase of biotinidase activity in individuals with GSD types III and IX (for example, heterozygosity for a mutation within the biotinidase gene), the *BTD* locus coding for biotinidase was analysed in three of the patients (Table 3, GSD III, patients 1 and 3; GSD IX, patient 2). It can be calculated from current screening programmes that carrier frequency for biotinidase deficiency in the general population is about one in 70 (Nennstiel-Ratzel et al 2004). However, while all mutated alleles could be identified in five children with biotinidase deficiency upon sequence analysis (data not shown), we have not detected any mutations in the *BTD* loci of the three GSD patients. Certainly, the number of patients tested was small, transcriptional products were not determined, and the patients' parents were not investigated for intermediate enzyme activities. Thus, an additional, independent genetic cause of inappropriate low biotinidase activity in a fraction of patients with GSD III and IX cannot ultimately be excluded.

Individual biochemical features

In Table 2 we summarize the individual biochemical features of the GSD and FBS patients from our study. Depending on the data available, liver glycogen content, residual enzyme activities and/or the affected gene loci are given. Serum biotinidase activity was independent of the glycogen content in liver. In addition, upon repeated measurements in a subset of patients, we observed no correlation between biotinidase activity, age and/or dietary control.

Table 4 The effect of freeze-thawing on serum biotinidase activity

Samples	*n*	Freeze-thaw cycles		
		1×	2×	3×
Control	6	100%	97%	92%
GSD Ia	2	100%	94%	90%

Biotinidase activity was measured at the designated freeze-thaw cycles. The measurements were done in triplicates. The relative percentages of biotinidase activities are given. The first freeze-thaw cycle (1×) represents the standard procedure (see "Methods") and is defined as 100%.

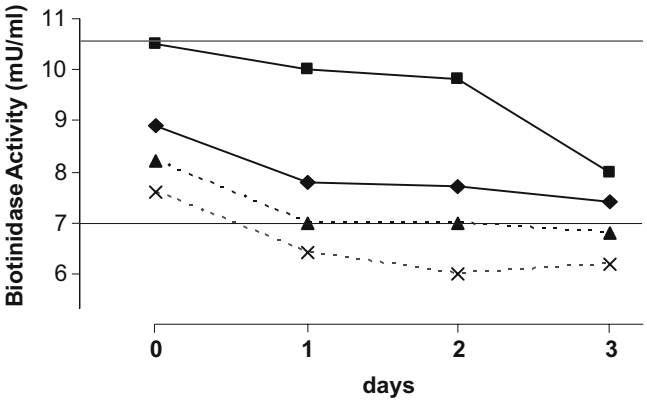

Fig. 2 Influence of blood sample storage on biotinidase activity. Samples from one healthy control were stored at room temperature as whole blood (-■-), serum (-◆-), heparin blood (-▲-), and plasma (-×-). Biotinidase activity was determined at days 0, 1, 2 and 3. The horizontal lines represent the range in sera of controls (7.0–10.6 mU/ml). The measurements were done in triplicates and mean values are shown

We identified one case with a 'false-positive biotinidase test', i.e., an elevated activity of 14.1 mU/ml in a patient with cystic fibrosis (OMIM 602421) who had massive hepatic steatosis. One of the 'false-negative biotinidase tests', namely normal serum biotinidase activity (9.6 mU/ml), was observed in a GSD type III patient with massive liver cirrhosis (Table 3, patient 5). Restricted biotinidase increase in this patient was most probably the result of liver biosynthesis failure. It has been reported previously that serum biotinidase activity is significantly lower in children with impaired hepatocellular function (Pabuccuoglu et al 2002), implying that it is important to rule out chronic liver parenchymal damage before applying the biotinidase test in a patient with presumed GSD.

Biotinidase activity in contrast patients

In order to test whether accumulation of other metabolic intermediates in liver can trigger upregulation of biotinidase activity, we measured biotinidase in sera from patients affected by lysosomal storage disorders (LSDs), including Gaucher disease (OMIM 230800) and Niemann–Pick disease type A (OMIM 257200) and type C (OMIM 257220). These disorders are characterized by deficient degradation of sphingolipids. In our patient group, they led to hepatosplenomegaly and mildly elevated transaminases but no chronic parenchymal destruction or cirrhosis. Serum biotinidase activity was normal in all these patients (Fig. 1) which argues against a non-specific phenomenon.

Blood sample handling and processing

We also assessed the effects of blood sample handling and processing on serum biotinidase activity, such as storage, freeze-thawing and transport delay. No significant differences in enzyme activity upon storage of serum samples at −20°C for up to one month were observed (data not shown). Next, serum aliquots from healthy controls and two unrelated GSD I patients underwent three freeze-thaw cycles and the activity was determined after each cycle (Table 4). The first freeze-thaw cycle represented 100% activity, according to our standard procedure (see "Methods"). After three freeze-thaw cycles the drop in biotinidase activity was about 10%. To investigate transport delays on biotinidase activity, blood from one healthy individual was stored for different periods at room temperature as native blood, heparinized blood, serum or plasma. As shown in Fig. 2, the enzymatic activities at day 0 were quite variable, with the highest activity measured in serum (10.5 mU/ml) and lowest in plasma (7.6 mU/ml). Over time, decrease in biotinidase activity in all samples was about 20%. These results suggest that biotinidase activities in plasma and serum samples, isolated at different time points, are not directly comparable. This fact, together with the less stringent sensitivity, might explain the normal biotinidase activities for GSD type III and IX patients reported elsewhere (Burlina et al 1996).

Our data show that hepatic glycogen storage diseases caused by deficiencies of enzymes involved in glycogen degradation lead to elevated biotinidase activity in blood. Even though this phenomenon can be used as a diagnostic tool, we can only speculate about the mechanism. A key function of biotinidase is to cleave biotin from biocytin, preserving the pool of biotin for use as a co-factor for biotin-dependent enzymes (Hymes and Wolf 1996). We therefore assume that metabolic pathways in these patients induce changes in such a way, that the demand for biotin as enzymatic co-factor is rigorously increased.

In summary, our study revealed significantly elevated biotinidase activities in sera from patients with GSD Ia and I non-a, GSD III, VI and IX and FBS. Thus, given that blood samples are processed properly, serum biotinidase serves as a simple, non-invasive tool to narrow down the differential diagnosis of hepatomegaly prior to specific enzymatic studies and/or mutation analysis.

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